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(54) Title: CROSS-REACTIVE INFLUENZA A IMMUNIZATION			
(57) Abstract This disclosure relates to methods and compositions for stimulating in an individual an influenza A virus protective response which is subtype cross-protective. Influenza A virus NS1 protein, or a T cell epitope thereof, is administered to the individual in an amount sufficient to stimulate the virus protective response.			

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CROSS-REACTIVE INFLUENZA A IMMUNIZATIONBackground of the Invention

Influenza A virus is a large RNA-containing animal virus. The protein capsid of the virus is further enclosed in a lipid bilayer-based envelope containing protruding spikes of viral glycoprotein. Three influenza A serotypes have been identified (H1, H2 and H3); the classification based upon differences in the viral glycoprotein.

Upon infection by the Influenza A virus, the body produces antibodies to variable regions of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). This response results in the production of virus-specific antibodies which constitute the primary defense of the immune system. These antibodies provide immunological pressure which leads to antigenic drift within viral subtypes, as well as shifts between viral subtypes. This relatively high rate of mutagenesis can render vaccine preparations ineffective because the antigenic determinants of the mutated viral proteins can differ significantly from those of the protein used as immunogen resulting in the failure of the body to effectively deal with the infection.

Two central components of the immune system are the B cells and T cells, both of which are lymphocytes. The lymphocyte lineage diverges at the prelymphoblast stage into distinct sublineages. B cells produce and secrete antibody molecules; a process generally referred to as the humoral response. T cells are responsible for a variety of cellular responses referred to generally as cell mediated immune responses.

B cells develop antigen specificity even in the absence of antigen stimulation. It has been estimated,

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for example, that the preimmune repertoire of a mouse comprises a class having many millions of different antibody molecules. This preimmune repertoire is apparently large enough to insure B cell specificity for almost any potential antigenic determinant.

Current inactivated whole or subunit influenza vaccines provide B cell mediated (humoral) immunity in that they induce antibodies which are directed toward antigenic determinants of the surface glycoproteins of the virus. The first presentation of an influenza antigen to a B cell specific for the antigen (e.g., at the time of vaccination) results in the maturation of the B cell into a plasma cell which is highly specialized for antibody production. Upon a second encounter with the same antigen, a rapid and increased secondary response results. The foreign antigen is bound by the specific antibody followed by clearance of the bound antigen from the bloodstream.

However, in the case of influenza A, the production of virus-neutralizing antibodies provides immunological pressure which leads to antigenic drift within viral subtypes, as well as shifts between viral subtypes. Vaccines which are directed against antigenic sites do not elicit a broadly cross-reactive (i.e., protective against all influenza A virus subtypes) B cell response. Furthermore, the mutations which result from this immunological pressure can render current vaccines ineffective.

T cells comprise a class of cells which, although they do not produce circulating antibodies, do play a central role in the immune system. The T cell class includes helper T cells, cytotoxic T cells and suppressor T cells. Helper T cells function, in part, by augmenting the response of other lymphocytes. For example, helper T cells stimulate activated T lymphocytes, in addition to

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stimulating B cells activation, by secreting interleukins as well as other soluble factors. Cytotoxic T cells (also referred to as killer T cells), on the other hand, function by destroying cells marked with a particular antigen (e.g., cells infected by virus).

T cells are stimulated by the recognition of a T cell epitope, in combination with a class I or a class II major histocompatibility (MHC) antigen, on the surface of an antigen presenting cell. Macrophages belong to the class of antigen presenting cells. Macrophages are phagocytes which ingest foreign particles in the body. These cells are capable of ingesting even large microorganisms such as protozoa. Following ingestion, the antigen presenting cell digests the foreign particle and fragments of the foreign particle are displayed on the surface of the cell.

T cell epitopes differ fundamentally from B cell epitopes. B cell epitopes are antigenic determinants found in the native antigen molecule and not represented in the denatured antigen or fragments thereof. T cell epitopes, on the other hand, are found on unfolded molecules or fragments thereof. Furthermore, the T cell epitopes comprise helper T cell epitopes and cytotoxic T cells epitopes. These epitopes are thought to be contained by distinct, albeit possibly overlapping, portions of the antigen molecule.

Influenza A virus infection continues to cause epidemics of death and tremendous morbidity throughout the world today even though the etiological agent is known. A great deal of effort has been devoted to the development of a vaccine, to little avail. A need exists for an effective influenza A vaccination strategy which could provide cross-subtype immunity from Influenza A viral infection.

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Summary of the Invention

This invention relates to Applicants' finding that T cell epitopes of the influenza A NS1 protein are capable of stimulating an influenza A virus protective response, in an individual, which is subtype cross-protective. In a first aspect, the method comprises administering an effective amount of influenza A virus NS1 protein, in combination with a pharmaceutically acceptable carrier thereby stimulating a T cell response against an NS1 epitope in the individual resulting in an influenza A virus protective response which is subtype cross-protective. A homologue of the NS1 protein in which amino acids have been deleted, inserted or substituted without essentially detracting from the immunological properties thereof, is also effective for this purpose.

In another aspect, the invention relates to a method and composition for immunizing an individual against infection by influenza A virus subtypes by the administration of an effective amount of an influenza A virus T cell epitope in combination with a pharmaceutically-acceptable carrier, thereby stimulating a T cell response against the NS1 epitope in the individual resulting in an influenza A protective response which is subtype cross-protective. The T cell epitope can stimulate a cytotoxic T cell response, a helper T cell response, or both. A homologue of the NS1 T cell epitope in which amino acids have been deleted, inserted or substituted without essentially detracting from the immunological properties thereof, is also effective for this purpose.

The invention also relates to an essentially pure oligopeptide having an amino acid sequence corresponding to a T cell epitope of the influenza A NS1 protein. This T cell epitope can stimulate a cytotoxic T cell response, and/or a helper T cell response. Again, a homologue of

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the NS1 T cell epitope in which amino acids have been deleted, inserted or substituted without essentially detracting from the immunological properties thereof, is also effective for this purpose.

5 Also disclosed is a method for immunizing an individual against infection by influenza A virus subtypes comprising administering an effective amount of a recombinant virus which expresses the influenza A virus NS1 protein. The individual can also be immunized by
10 administering an effective amount of a recombinant virus which expresses an influenza A virus T cell epitope. These methods are limited to the administration of a recombinant virus which expresses the NS1 protein or a T cell epitope thereof, thereby stimulating a T cell
15 response against a T cell epitope resulting in an influenza A virus protective response which is subtype cross-protective.

The methods and compositions described herein provide for a broadly cross-reactive vaccination scheme which is
20 protective against H1, H2 and H3 subtypes of influenza A virus.

Detailed Description of the Invention

As discussed previously, influenza A virus comprises three subtypes, H1, H2 and H3. Applicants' invention
25 relates to methods for immunizing an individual, particularly a human, against infection by any of these subtypes. Although the methods described herein are particularly useful for human immunization, the methods are equally applicable to other mammals. The term "cross-
30 protective" is used in this application to describe immunity against H1, H2 and H3 subtypes.

The gene encoding the influenza A NS1 protein has been isolated, cloned, and expressed in a recombinant vaccinia system (see e.g., Bennink et al., *J. Virol.*

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61:1098-1102 (1987)). Using standard biochemical techniques (e.g., column chromatography) the NS1 protein, having a known molecular weight, can be isolated from cells in which it is expressed. If necessary to attain the desired purity, a hybridoma producing monoclonal antibody specific for NS1 can be generated. Monoclonal antibody produced by this hybridoma can then be used in an affinity capture purification scheme.

Homologues of the NS1 protein in which amino acids have been deleted, inserted or substituted without essentially detracting from the immunological properties thereof can be generated in a variety of ways. For example, *in vitro* mutagenic techniques can be used to modify the cloned gene encoding the NS1 protein. Such methods, which are well known to one skilled in the art, can be used to delete, insert or substitute nucleotides in the gene resulting in the deletion, insertion or substitution of amino acids in the encoded product. The immunological properties of the mutagenized encoded product can be assayed using methods such as those described in the Exemplification which follows.

Effective dosages for the stimulation of an influenza A virus protective response are determined empirically with initial dosage ranges based upon historical data for peptide/protein vaccine compositions. As used herein, the term virus protective refers to an immunological response in the individual resulting in the successful control or limitation of infection by influenza A virus subtypes which is clinically observed.

For example, individuals can be administered dosages of NS1 protein ranging from 0.5-500 micrograms. Whether a particular dosage is effective can be determined using well known T cell proliferation and cytotoxicity assays. For example, following administration of the protein to an individual blood is drawn. Cytotoxic T cells are

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identifiable by ^{51}Cr release assay (see e.g., Kuwano et al., *J. Virol.* 140:1264-1268 (1988)). Helper T cells are identifiable by a standard T cell proliferation assay (see e.g., Kurane et al., *J. Clin. Invest.* 83:506-513 (1989)).

- 5 The results from these studies are compared with results from the same experiments conducted with T cells from the same individual prior to administration of the antigen. By comparing this data, effective dosage ranges can be determined.

- 10 A wide variety of pharmaceutically acceptable carriers are useful. Pharmaceutically acceptable carriers include, for example, water, physiological saline, ethanol polyols (e.g., glycerol or administration is typically parenteral (i.e., intravenous, intramuscular,
- 15 intraperitoneal or subcutaneous). An adjuvant (e.g., alum) can also be included in the vaccine mixture.

- The invention also pertains to a method for immunizing an individual against infection by influenza A virus subtypes by administering a vaccine composition
- 20 comprising at least one essentially pure T cell epitope of the NS1 protein in combination with a pharmaceutically acceptable carrier. Due to genetic variability between individuals, a single T cell epitope may not stimulate a virus protective response in all individuals to whom it is
- 25 administered. Therefore, by combining two or more distinct T cell epitopes, the vaccine is more broadly effective. As indicated above, helper T cell epitopes and cytotoxic T cell epitopes are thought to comprise distinct (albeit possibly overlapping) regions of proteins.
- 30 Cytotoxic T cell epitopes can be distinguished from helper T cells epitopes experimentally using the cytotoxicity and proliferation assays described above (helper T cells stimulate proliferation but do not possess cytotoxic activity).

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The T cell epitope will be administered as an oligopeptide. Such oligopeptides can be synthesized chemically following identification of the portion of the protein containing the T cell epitope. Alternatively, a truncated portion of the gene encoding the NS1 protein which contains a T cell epitope can be expressed in a cell, and the encoded product can be isolated using known methods (e.g., column chromatography, gel electrophoresis, etc.). In addition, the intact NS1 protein can be treated chemically or enzymatically to generate fragments which contain a T cell epitope. Such fragments can be isolated as described above.

As used herein, the term oligopeptide means any amino acid sequence which is identical or substantially homologous to a portion of the NS1 protein. The expression substantially homologous refers to oligopeptides having an amino acid sequence of an NS1 T cell epitope in which amino acids have been deleted, inserted or substituted without essentially detracting from the immunological properties thereof. This definition includes amino acid sequences of sufficient length to be classified as polypeptides (these terms are not used consistently or with great precision in the literature).

In a preferred embodiment, both a helper T cell epitope and a cytotoxic T cell epitope are administered to the individual. The stimulation of cytotoxic T cells is desirable in that these cells will kill cells infected by influenza A virus. The stimulation of helper T cells is beneficial in that they secrete soluble factors which have a stimulatory effect on other T cells, as well as B cells. As discussed above, due to the genetic variability between individuals, it is preferable to include two or more cytotoxic T cell epitopes and two or more helper T cell epitopes.

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Several methods are described in the literature which are useful for the identification of T cell epitopes. For example, DeLisi et al. have suggested that potential epitopic sites may be located by identification of

5 potential amphipathic alpha helical regions in the molecule. DeLisi et al., *Proc. Natl. Acad. Sci. USA* 82:7048 (1987). Bixler et al. describe a strategy of synthesizing overlapping synthetic peptides encompassing an entire protein molecule for delineation of T cell

10 epitopes. Bixler et al., *Immunol. Com.* 12:593 (1983); Bixler et al. *J. Immunogenet.* 11:339 (1994). A synthetic method described by Gysen (*Ciba Foundation Symposium* 119:130 (1986)) permits synthesis of a large variety of peptides thereby mimicking of a variety of potential

15 binding sites, in turn allowing rapid scanning of a molecule.

More traditional methods, such as enzymatic or chemical digestion of proteins provide peptide fragments which may be readily tested for T cell activity. For

20 example, enzymes such as chymotrypsin, elastase, ficin, papain, pepsin, or trypsin provide limited and predictable fragments by cleavage of specified amino acid linkages; similarly chemical compounds such as N-chloro-succinimide BPNS-skatole, cyanogen bromide, formic acid, or

25 hydroxylamine, also produce definable fragments by their action on proteins. The presence of the desired T cell stimulating activity in any given fragment can be readily determined by subjecting purified fragments to a standard T cell proliferation assay, or by analyzing unpurified

30 fragments with a T cell Western Assay. Young et al., *Immunol.* 59:167 (1986).

In another embodiment, the gene encoding the NS1 protein, or a portion thereof which contains a T cell epitope, can be cloned into a recombinant virus which

35 expresses the NS1 protein, or T cell epitope containing

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portion thereof, in the individual to be immunized. An example of such a recombinant virus system is the vaccinia system described by Paoletti et al. (U.S. Patent No. 4,603,112), the disclosure of which is incorporated herein by reference. Other viruses have been described in the literature which have a genome which can accommodate the insertion of a foreign DNA such that a protein encoded by the DNA is expressed in vivo. Any such recombinant virus is useful for the practice of this invention.

One skilled in the art will recognize that the compositions described herein can be combined with the components of influenza A vaccines currently in use, thereby resulting in an improved vaccine. The invention is illustrated further by the following Exemplification.

EXEMPLIFICATION

Example 1: Adoptive Transfer In Mice

Mice

BALB/c mice (H-2^d) were purchased from Charles River Breeding Laboratories (Stone Ridge, NY). They were used at 5 to 9 weeks of age.

Influenza Viruses

Influenza A viruses, A/PR/8 (H1N1), A/BZ (H1N1), A/JAP (H2N2), and A/PC (H3N2), or B/HK, were propagated in 10-day-old embryonated chicken eggs. Infected allantoic fluids were harvested 2 days after infection, aliquoted, and stored at -80° (Kuwano, K. et al., *J. Immunol.* 140:1264-1268 (1988)).

Vaccinia Viruses

Vaccinia recombinant viruses containing genes (HA, NP, NS1, and PB2) for A/PR/8 virus were kindly provided by

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Dr. B. Moss (Bethesda, MD). They were constructed and propagated as previously described (Smith, G.L. et al., *Virology* 160:336-345 (1987)). Briefly, HeLa cells were infected with virus for 3 days at 37°. Infected cells
5 were pelleted by centrifugation, and resuspended in MEM containing 2% FCS. Three cycles of freezing and thawing were performed and the suspensions were gently sonicated in water for 1 min followed by trypsinization for 30 min at 37°. After centrifugation at 500 rpm for 5 min,
10 supernatants were aliquoted and stored at -80°.

Cells

The cell lines used in this study, P815 cells (H-2^d; mastocytoma) derived from DBA/2 mice, Class 1 MHC molecules, H-2L^d- or H-2D^d-transfected L929 cell line, and
15 LM1 (K^k, L^d, D^k) or DM1 (K^k, L^k, D^d), were as described by Weis, J.H. and J.G. Seidman (*J. Immunol.* 134:1999-2003 (1985)).

Fusion Protein

D proteins were produced in *E. coli* as described
20 previously (Yamada, A. et al., *Escherichia coli. J. Exp. Med.* 162:663-674 (1985); Kuwano, K. et al., *J. Immunol.* 140:1264-1268 (1988)). Briefly, plasmids containing DNA fragments complementary to the viral RNA of A/PR/8 virus were manipulated to achieve expression of D
25 proteins, which are hybrids of the first 81 amino acids of NS1 fused to the 157 amino acids from the C-terminal end of HA2 through a linker of glutamine-isoleucine-proline. After lysis of the bacteria, two 0.1% deoxycholate extractions and one 1% Triton X-100 extraction were
30 performed to remove contaminating *E. coli* proteins, and the D protein was solubilized with 4 M urea at 4° for 30 min. The urea was removed by dialysis at 4°. The proteins were stored in 50 mM Tris-HCl, pH 8.0, and 1 mM

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EDTA. D protein was provided by J.F. Young (Smith, Kline and French Laboratories, Philadelphia, PA).

CTL Clone

CTL clones were established as described previously (Kuwano, K. et al., *J. Immunol.* 140:1264-1268 (1988)). Briefly, CTL responder cells were stimulated weekly with A/PR/8 or D protein-pulsed normal syngeneic γ -irradiated spleen cells in the presence of 10% Con A stimulated rat IL2 for several weeks. A limiting dilution was carried out to isolate CTL clones. The B-7 clone was established by stimulation of D protein (Kuwano, K. et al., *J. Immunol.* 140:1264-1268 (1988)). The A-11 clone was stimulated by A/PR/8 virus and grew, at a frequency of growth 2 out of 96 wells, from a well where two responder cells had been seeded. For routine passage of clones, 2 X 10⁶ of clone cell were stimulated weekly by 30 X 10⁶ of A/PR/8 virus or D protein treated γ -irradiated spleen cells in the presence of 10% rat IL2 and 5 X 10⁻⁵ M 2-ME.

CTL Assay

P815, LM1, or DM1 cells (2 X 10⁶) were incubated with 0.5 ml of virus (10⁷ -10⁸ PFU) in the presence of ⁵¹Cr at 37° for 60 min. After three washings, target cells were incubated for another 1 hr. Then 1 X 10⁴ ⁵¹Cr-labeled target cells were incubated with 1 X 10⁴ effector cells in a total volume of 200 μ l in 96-well round bottom microplates for 4 hr at 37°. The supernatant fluids were harvested and specific lysis was determined as percentage specific lysis = 100 X [(release by CTL - spontaneous release)/(maximum release - spontaneous release)].

Adoptive Transfer of CTL Clone

Cells of the CTL clone (3 X 10⁶) were suspended in 0.5 ml of RPMI 1640 and injected into mice via the tail

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vein. A preliminary experiment indicated that transfer of 1.0×10^6 cells resulted in significant reductions in mean pulmonary virus titers ($0.6 - 0.8 \log_{10}$ PFU) in recipients of clone A-11. Six hours after adoptive transfer of the CTL clone, mice were infected intranasally with 10^3 PFU of virus under ether anesthesia. The lungs of four mice per group were harvested 3 days later for measurement of virus titers.

Pulmonary Virus Titrations

10 Virus titrations were performed by plaque formation using MDCK cells as previously described (Kuwano, K. et al., *J. Immunol.* 140:1264-1268 (1988)). Briefly, infected lungs taken from recipient mice were manually homogenized in 1.5 ml of PBS containing 0.1% BSA. After
15 centrifugation, the lung supernatants were serially 10-fold diluted in PBS. Diluted virus samples (100 μ l) were added to confluent MDCK cells in 24-well tissue culture plates and incubated at 37° for 1 hr. Each well then received 1 ml of 1% agar prepared as described earlier
20 (Kuwano, K. et al., *J. Immunol.* 140:1264-1268 (1988)). After 2 days of incubation, 1 ml of 10% neutral red (GIBCO, Chagrin Falls, OH) in PBS was overlaid on the agar in the wells. Plaques were counted 8 hr later. The results were expressed as the mean \log_{10} PFU/ml of
25 duplicate samples.

Cross-Reactivity of Clone A-11 Stimulated by A/PR/8 Virus

Four CTL clones were established that were derived from A/PR/8 virus-immune spleen cells of BALB/c mice (H-2^d) stimulated by A/PR/8 virus (H1N1). Two of the CTL
30 clones demonstrated H1 subtype-specific lysis of virus-infected target cells. These CTL clones were PB2 protein specific as determined using target cells infected with a

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vaccinia recombinant virus containing the gene for PB2 of A/PR/8 virus. Clone 1E8, representative of two subtype H1-specific clones, is shown as a negative control in Table 2. Clone A-11, which is representative of the other two CTL clones, demonstrated cross-reactive lysis of target cells which were infected with A/PR/8 (H1N1), A/BZ (H1N1), A/JAP (H2N2), or A/PC (H3N2) viruses, but failed to lyse B/HK-infected target cells (Table 1). The B-7 CTL clone (Kuwano, K. et al., *J. Immunol.* 140:1264-1268 (1988)) was used as a control. B-7 had been stimulated by a fusion protein containing part of the HA2 subunit of A/PR/8 virus and showed subtype H1H2 cross-reactive lysis of target cells that had been infected with A/PR/8 (H1), A/BZ (H1), or A/JAP (H2) viruses. The phenotypes of the cell surface antigens of both the A-11 and B-7 clones were Thyl+, Lyt-2+, and L3T4.

TABLE.1
Virus Specificity of Clone A-11 Stimulated by
A/PR/8 Virus

		<u>% Specific Lysis of PB15 Target Cells</u>					
		A/PR/8	A/BZ	A/JAP	A/PC		
<u>Clone</u>	<u>E/T Ratio</u>	<u>(H1N1)</u>	<u>(H1N1)</u>	<u>(H2N2)</u>	<u>(H3N2)</u>	<u>B/HK</u>	<u>Uninfected</u>
A-11 ^a	1.0	54	59	58	62	0	0
	0.5	43	51	43	43	0	0
B-7 ^b	1.0	62	65	43	0	1	0
	0.5	45	52	32	0	0	0

^aClone A-11 expresses 94% of Thy1.2, 86% of Lyt-2, and 5% of L3T4 surface Ag.

^bClone B-7 expresses 97% of Thy1.2, 95% of Lyt-2, and 0% of L3T4 surface Ag.

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CTL Clone A-11 is NS1-Protein Specific

To examine the influenza protein recognized by clone A-11, target cells were infected with recombinant vaccinia viruses containing various influenza genes of A/PR/8 virus and were used in CTL assays. As shown in Table 2, clone A-11 significantly lysed NS1-VAC-infected and A/PR/8 virus-infected P815 target cells as positive control. However, clone A-11 failed to recognize HA-VAC, NP-VAC, PB2-VAC, or parental VAC-infected P815 target cells.

Clone B-7 as a negative control lysed HA-VAC-infected target cells as well as A/PR/8 virus-infected target cells, but did not lyse NP-VAC-or VAC-infected target cells. Clone 1E8, also derived from A/PR/8 virus-immune spleen cells by repeated stimulation with A/PR/8 virus as described above, recognized PB2-VAC-infected target cells or A/PR/8 virus-infected target cells, but failed to recognize NS1-VAC or HA-VAC-infected target cells; it is also included as a control. These results indicated that CTL clone A-11 recognizes the NS1 protein on influenza A virus-infected cells.

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TABLE 2
Recognition of NS1 Protein of A/PR/8
Virus by Clone A-11

% Specific Lysis of P815 Target Cells

<u>Clone</u>	<u>E/T</u> <u>Ratio</u>	<u>A/PR/8</u>	<u>HA-VAC</u>	<u>NP-VAC</u>	<u>NS1-</u> <u>VAC</u>	<u>PB2-</u> <u>VAC</u>	<u>VAC</u>	<u>Unin-</u> <u>fectd</u>
Experiment 1								
A-11	1.0	55	-4	ND	42	-4	ND	-1
	0.5	47	-4	ND	33	-2		-2
1E8	1.0	61	-3	ND	0	62	ND	-1
	0.5	53	-4	ND	0	47	ND	-1
Experiment 2								
A-11	3.0	78	0	-1	ND	ND	0	1
	1.0	75	1	-1	ND	ND	0	1
B-7	3.0	91	91	-1	ND	ND	-2	0
	1.0	72	80	-1	ND	ND	-1	0

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Reduction of Pulmonary Virus Titers by Transfer of NS1-Specific CTL Clone

To examine whether adoptive transfer of NS1 protein-specific CTL clone A-11 would reduce virus titers in the lungs of mice infected with influenza viruses, 3×10^6 cells of clone A-11 were adoptively transferred to BALB/c mice 6 hr prior to influenza infection. Three days later, lungs were removed for titration of influenza viruses. Virus titrations were performed by plaque formation assays in MDCK cells. Similar results were obtained in two experiments with mean decreases in pulmonary virus titers of about $1.0 \log_{10}$. As shown in Table 3, adoptive transfer of CTL clone A-11 significantly reduced the virus titers in the lungs of mice infected with A/PR/8, A/JAP, or A/PC viruses, but did not reduce the virus titer in the lungs of mice infected with B/HK virus. These results reflect the *in vitro* cross-reactivity of CTL clone A-11 shown in Table 1.

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TABLE 3
Reduction of Pulmonary Virus Titers by
Adoptive Transfers of Clone A-11

<u>CTL</u> <u>Clone A-11^a</u>	<u>Virus</u> <u>Challenge</u>	<u>Virus Titer in Lungs^b</u>		<u>RECIPIENTS</u>
		<u>Experiment 1</u>		<u>Experiment 2</u>
+	A/PR/8 (N1N1)	5.1±0.4 ^c		5.7±0.2 ^f
-	A/PR/8	6.2±0.3		6.8±0.2
+	A/JAP (H2N2)	3.0±0.6 ^d		3.3±0.2 ^g
-	A/JAP	4.3±0.2		4.4±0.2
+	A/PC (H3N2)	4.0±0.4 ^e		4.5±0.4 ^h
-	A/PC	5.0±0.1		5.7±0.2
+	B/HK	4.1±0.1		ND
-	B/HK	4.2±0.1		

^aCells (3 x 10⁶) were transferred 6 hr before virus challenge; +, transferred; -, no cells transferred.

^bLungs were taken and virus titers were examined by plaque assays in MDCK cells 3 days after virus challenge.

^cp<0.01, Student's t test.

^dp<0.02, Student's t test.

^ep<0.005, Student's t test.

^fp<0.005, Student's t test.

^gp<0.0005, Student's t test.

^hp<0.005, Student's t test.

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MHC Restriction of Target Cell Lysis by Clone A-11

L929 cells (H-2^k) transfected with genes encoding H-2D^d (DM1 cells) and H-2L^d (LM1 cells) were used to examine the MHC restriction of target cells lysis by CTL clone A-11. As shown in Table 4, CTL clone A-11 significantly lysed A/PR/8 virus-infected LM1 (H-2L^d) target cells, but failed to lyse A/PR/8 virus-infected DM1 (H-2^d) or A/PR/8 virus-infected DAP (H-2^k) target cells.

As a control, CTL derived from bulk cultures of A/PR/8 virus-immune BALB/c (H-2d) spleen cells that had been stimulated by A/PR/8 virus in the presence of IL2 for several weeks were also used in this experiment. These virus-stimulated CTL lysed LM1 or DM1 target cells infected with A/PR/8 virus, but did not kill A/PR/8 virus-infected DAP target cells. It was also observed that the CTL clone A-11 was unable to recognize A/PR/8 virus-infected peritoneal exudate cells of C3H.OL mice (H-2K^d, D^k). These results indicate that recognition by the CTL clone A-11 of NS1 on A/PR/8 virus-infected target cells is restricted by the H-2L^d allele.

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TABLE 4
MHC Restriction of CTL Recognition by
CTL Clone A-11

<u>% Specific Lysis of Target Cells</u>							
<u>CTL</u>	<u>E/T</u>	<u>LM1 (H-2K^k, D^k, L^d)</u>		<u>DM1 (H-2K^k, D^k, D^d)</u>		<u>DAP (H-2K^k, D^k)</u>	
	<u>Ratio</u>	<u>A/PR/8</u>	<u>None</u>	<u>A/PR/8</u>	<u>None</u>	<u>A/PR/8</u>	<u>None</u>
A-11	5.0	58	1	9	2	0	0
	2.5	43	1	2	2	2	0
A/PR/8							
stimu-							
lated							
CTL	5.0	36	2	24	2	1	1
	2.5	30	1	11	1	1	1

EXAMPLE 2: Active Immunization In Mice

Active Immunization with the Recombinant Vaccinia Virus
Expressing NS1 CTL Epitope is Protective in Mice

In Example 1, results were presented that

5 demonstrated an influenza A NS1 cross-reactive cytotoxic T lymphocyte response in H-2^d mice. The ability of an NS1 specific CTL clone to passively protect mice after adoptive transfer of the NS1 specific CTL clone was reported. There were significant decreases in lung virus

10 titers of mice challenged with influenza A viruses of all three subtypes i.e., H1, H2 and H3. The influenza A NS1 gene was the kind gift of Dr. Robert Lamb of Northwestern University and the gene was truncated and expressed in vaccinia virus. The results shown in Table 5 indicate

15 that a CTL epitope recognized by an influenza A NS1 cross-reactive CTL clone was located between amino acids 1-40 of NS1.

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TABLE 5

Recognition of Epitope(s) on Influenza A Non-Structural
Protein by Virus-Stimulated CTL from BALB/c Virus-Immune
Spleen Cells in Bulk Culture

	<u>Percent specific lysis at E:T</u>		
Target Cells Infected With:			
	<u>20.0</u>	<u>100</u>	<u>50</u>
A/PR/8	71.9	56.9	47.2
VAC-entire NS1	74.1	40.3	14.9
VAC2 (aal-167)	41.0	25.2	12.3
VAC10 (aal-40)	43.5	19.4	16.2
Vaccinia alone	-1.6	5.0	4.3
Uninfected	13.7	6.5	4.9

The results in Table 5 demonstrate that as a positive control virus-stimulated immune spleen cells killed virus-infected target cells and did not significantly kill uninfected target cells; moreover, the results demonstrate

5 that target cells infected with recombinant vaccinia viruses expressing various regions of the NS1 gene including the VAC-10 recombinant with amino acids 1-40 were significantly killed by the influenza virus stimulated PBMC. These results indicate that following a

10 single infection with influenza A virus, significant memory CTL activity to epitopes on influenza A NS1 is present in H-2^d mice.

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Active Immunization with VAC/NS1 Recombinant Virus

It was important to determine if it was possible to actively immunize mice with a recombinant virus containing an NS1 gene segment to protect against influenza virus.

5 An NS1-specific cross-reactive CD8+ CTL responses had been detected in bulk cultures and at the clonal level, and protection in passive immunization studies with an NS1-specific clone had been shown, but active immunization with a vaccine had not been carried out.

10 Mice were immunized with 0.2ml (1×10^8 PFU/ml) of a control vaccinia virus or the recombinant vaccinia-10 which contained the gene segment encoding influenza A NS1 amino acids 1-40. A second dose of 0.2ml with the same amount of virus was administered IP to the two groups of
15 mice three weeks later. Five weeks after the initial dose and two weeks following the second dose spleen cells were removed, restimulated in vitro with A/PR/8 and then used in a CTL assay.

The results in Table 6 indicate that influenza A
20 virus-specific CTL memory was induced following administration with the vaccinia-10 recombinant and boosting in vitro with influenza A virus. These cells killed influenza A virus infected target cells, and uninfected target cells were not lysed. The spleen cells
25 of the control mice that were primed with vaccinia virus not containing the NS1 gene, were restimulated with live influenza virus but their cells did not specifically lyse influenza A virus infected target cells. These results indicate that the priming of influenza A virus NS1
30 specific T cells resulted in the subsequent generation of influenza A virus cytotoxic T lymphocytes following repeat stimulation with influenza A virus.

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TABLE 6

Vaccinia Virus Containing Gene Encoding NS1 aal-40 Induces
Influenza Virus CTL Memory in BALB/c (H-2^d) Mice

Lysis of H-2^d Target Cells

1°	2°		A/PR/8	
<u>In Vivo</u>	<u>In Vitro</u>	<u>E:T</u>	<u>infected</u>	<u>Uninfected</u>
VAC-10	A/PR/8	30	11.2	-1.3
(NS1-		10	-1.0	4.4
segment)				
Vaccinia	A/PR/8	30	-1.0	1.6
		10	1.8	5.6

Seven weeks after the original immunization, mice in these groups were challenged with 4×10^3 plaque forming units of virulent influenza A/PR/34 (H1N1) virus administered in 50 μ l volumes intranasally. The results from these experiments demonstrated that mice immunized with VAC/NS1 were significantly protected against lethal challenge with influenza A virus compared to mice given vaccinia virus alone ($P < 0.05$). More specifically, the survival rate of VAC/NS1 immunized mice observed at 9-16 days post-infection was between 70-80%. The survival rate of mice infected with vaccinia virus alone (negative control) was about 20% during the same time period. It was important to determine if active immunization would induce protection against disease and death because Stitz et al. reported that a recombinant vaccinia virus which expressed the nucleoprotein (NP) gene of influenza A virus contained a CTL epitope did not protect when a recombinant vaccinia virus containing the gene for influenza nucleoprotein was used to immunize mice, even though a CTL

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clone specific for NP epitope was protective in passive transfer experiments.

The nucleoprotein gene induced influenza A virus memory CTL's in vaccinated mice but did not induce protection against subsequent infection(s). The results presented herein demonstrate that mice which had received the vaccinia expressing a segment of the influenza A NS1 gene had CTL memory responses and were protected against lethal challenge. In conclusion, these results demonstrate that influenza A NS1 virus contains protective CTL epitope(s) and can be used as a vaccine to protect against influenza mortality in mice. The results below indicate that humans also have influenza A NS1-specific CTL's.

15 EXAMPLE 3: Generation & Characterization of a Human Influenza NS1-Specific T Cell Line

Method of Establishing Human NS1-Specific T Cell Line #77

Human PBMC were stimulated in bulk culture by exposure to influenza A/PR/8/34 (H1N1) infected autologous stimulator cells (1.6×10^6) which had been infected with virus at a multiplicity of infection of 10 to 1 for ninety minutes. After two washes these cells were added to 7×10^6 responder PBMC in AIM V medium containing 15% human AB serum. Following six days of culture, the cells were tested for their ability to lyse autologous EBV-transformed B cells in a standard 51 chromium release assay. Table 7 demonstrates results at two effector: target ratios, 50 and 10:1. There is specific lysis of the autologous target cells infected with influenza virus.

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TABLE 7Percent Specific Lysis of Target Cells at E:T Ratio

<u>Effector cells</u>	<u>Uninfected</u>		<u>A/PR/8 Infected</u>	
A/PR/8 Stimulated	<u>50</u>	<u>10</u>	<u>50</u>	<u>10</u>
	10.7	7.9	45.6	33.9

On day 8, cells from the original culture were re-stimulated with x-irradiated autologous PBMC which had been infected with influenza A virus, similar to the methods used on day 0. On day 14, a limiting-dilution was performed and 60 wells each received either 10 cells, or 30 cells per well in AIM V media, with 10% fetal calf serum 10% T cell growth factor 0.1 microgram per ml of anti-CD3 mab and x-irradiated autologous PBMC cells. Fifteen days later, positive growth was seen in 27 of the 60 wells that were seeded at 10 cells per well, and 52 wells of the 60 that were seeded at 30 cells per well. Three wells were positive in cytotoxicity experiments and the cells in well #77 lysed influenza virus infected autologous BLCL but has very little lytic activity on the human NK sensitive cell K562 (13.1% vs. 2.1%). The next day, the cells in well #77 were fed and expanded by being re-stimulated every 14 days with the addition of x-irradiated PBMC and anti-CD3 antibody in the presence of AIM V medium and 10% fetal bovine serum and 10% T cell growth factor. The results in Table 8 show the killing activity of cells from well #77 on autologous BLCL target cell infected with the vaccinia/NS1 recombinant virus that contained the NS1 gene of influenza A virus infected with influenza A virus A/PR/8/34, but they did not kill target cells infected with vaccinia virus along or vaccinia viruses expressing the influenza A virus hemagglutinin gene, the nucleo protein gene. The results indicate that

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cells from well #77 specifically killed autologous target cells that expressed the influenza A NS1 gene.

TABLE 8

Lysis of Autologous B-LCL Target Cells Infected with
Recombinant Vac/Influenza Viruses by Clone/Line #77

Effector Cells							Unin-
#77	ET	A/PR/8	VAC	VAC HA	VAC NP	VAC NS1	fected
	10	55:0	-4.9	-7.9	-11.4	32.1	-19.5

Three weeks later, the T cell line #77 was tested on target cells infected with an influenza A (A/PC/1/73, H3 subtype) virus. These target cells were also killed to a high degree, as were target cells infected with the

5 vaccinia recombinant virus expressing the NS1 truncated gene, but #77 cells did not kill target cells infected with recombinant vaccinia viruses expressing the other genes, hemagglutinin or nucleoprotein, of the influenza A virus. These results demonstrate that clone/line #77

10 specifically kills autologous cells expressing epitope(s) localized within amino acids 1-167 of the influenza A NS1 protein.

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TABLE 9

Specific-Lysis by Clone #77 of Autologous B-LCL Infected
with Vaccinia Expressing Truncated Influenza A Virus NS1
Gene Segments

Percent specific-lysis of target cells

Effector Cells					Influenza
#77	ET	VAC-entire NS1 1-237aa	VAC-NS1 (aal-167)	VAC-NS1 (aal-81)	A/PC/73 (H3N2)
	10	71.4	67.1	34.4	75.2

HLA Restriction of Influenza A NS1 Specific Cytotoxic T
Lymphocyte Activity

Experiments were performed using allogeneic target cells which contained partially matched HLA alleles to define the HLA restriction allele used by the NS1 specific T cell line #77. The results of the first experiment shown in Table 10 demonstrate that allogeneic target cells that share HLA A1 and B8 alleles were killed and that target cells that shared B44 or CW5 were not killed. In the second experiment partially matched B-LCL target cells from three different donors were used, two of which shared only A1 and one of which shared only the B8 allele. The results demonstrate that allogeneic target cells bearing the HLA B8 allele are killed, but those that shared only A1 were not. In summary, these results clearly demonstrate the existence of a human Class I restricted, influenza A virus NS1 subtype cross-reactive cytotoxic T lymphocyte responses.

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TABLE 10

HLA Class I Restriction of #77, NS1-Specific
Human CTL Percent Specific Lysis of HLA
Partially Matched Cells

A.

HLA Shared	VAC-/NS1 (entire)
Autologous	
(A1 B8 B44 CW5)	69.7
A1 B8	64.0
B44 CW5	-7.1
A1 B8	73.3

B.

Autologous	78.5
A1	-2.1
B8	59.6
A1	-0.7

Equivalents

Those skilled in the art will know, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and other equivalents are intended to be encompassed by the following claims.

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CLAIMS

1. A method for immunizing an individual against infection by influenza A virus subtypes comprising administering to the individual, in combination with
5 a pharmaceutically acceptable carrier, an effective amount of influenza A virus NS1 protein, or an influenza A virus NS1 protein derivative in which one or more amino acids have been deleted, inserted or substituted without essentially detracting from the
10 immunological properties thereof wherein said NS1 protein or NS1 protein derivative stimulates a T cell response against an NS1 epitope.
2. The method of Claim 1 wherein said NS1 protein derivative consists essentially of the NS1 epitope.
- 15 3. The method of Claim 2 wherein said NS1 epitope stimulates a cytotoxic T cell response.
4. The method of Claim 2 wherein said NS1 epitope stimulates a helper T cell response.
5. The method of Claim 1 wherein said NS1 protein
20 derivative consists essentially of amino acids 1-167 of NS1 protein.
6. The method of Claim 1 wherein said NS1 protein derivative consists essentially of amino acids 1-40 of NS1 protein.
- 25 7. A method for immunizing an individual against infection by influenza A virus subtypes comprising administering to the individual an effective amount of a recombinant virus which expresses influenza A

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- 5 virus NS1 protein, or an influenza A virus NS1 protein derivative in which amino acids have been deleted, inserted or substituted without essentially detracting from the immunological properties thereof, wherein said NS1 protein or NS1 protein derivative stimulates a T cell response against an NS1 epitope.
8. The method of Claim 7 wherein said NS1 protein derivative consists essentially of the NS1 epitope.
- 10 9. The method of Claim 8 wherein said NS1 epitope stimulates a cytotoxic T cell response.
10. The method of Claim 8 wherein said NS1 epitope stimulates a helper T cell response.
- 15 11. The method of Claim 7 wherein said NS1 protein derivative consists essentially of amino acids 1-167 of NS1 protein.
12. The method of Claim 7 wherein said NS1 protein derivative consists essentially of amino acids 1-40 of NS1 protein.
- 20 13. A vaccine composition, in combination with a pharmaceutically acceptable carrier, comprising an effective amount of an influenza A virus NS1 protein derivative wherein one or more amino acids have been inserted, deleted or substituted without detracting from the immunological properties thereof, wherein
25 said derivative stimulates a T cell response against an NS1 epitope when administered to an individual.

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14. The vaccine composition of Claim 13 wherein said NS1 protein derivative consists essentially of the NS1 epitope.
15. The vaccine composition of Claim 14 wherein said NS1 epitope stimulates a cytotoxic T cell response.
16. The vaccine composition of Claim 14 wherein said NS1 epitope stimulates a helper T cell response.
17. The vaccine composition of Claim 13 wherein said NS1 protein derivative consists essentially of amino acids 1-167 of NS1 protein.
18. The vaccine composition of Claim 13 wherein said NS1 protein derivative consists essentially of amino acids 1-40 of NS1 protein.
19. A vaccine composition, in combination with a pharmaceuticlaly acceptable carrier, comprising an effective amount of a recombinant virus which expresses influenza A virus NS1 protein or derivative thereof wherein one or more amino acids have been inserted, deleted or substituted without detracting from the immunological properties thereof, wherein said NS1 protein or NS1 protein derivative stimulates a T cell response against an NS1 epitope resulting in an influenza A virus protective response when administered to an individual.
20. The vaccine composition of Claim 19 wherein said NS1 protein derivative consists essentially of the NS1 epitope.

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21. The vaccine composition of Claim 20 wherein said NS1 epitope stimulates a cytotoxic T cell response.
22. The vaccine composition of Claim 20 wherein said NS1 epitope stimulates a helper T cell response.
- 5 23. The vaccine composition of Claim 19 wherein said NS1 protein derivative consists essentially of amino acids 1-167 of NS1 protein.
24. The vaccine composition of Claim 19 wherein said NS1
10 acids 1-40 of NS1 protein.
25. A recombinant virus which expresses an influenza A virus NS1 protein derivative wherein one or more amino acids have been inserted, deleted or substituted without detracting from the immunological
15 properties thereof wherein said NS1 protein derivative stimulates a T cell response against an NS1 epitope.
26. The recombinant virus of Claim 25 wherein said NS1 protein derivative consists essentially of the NS1
20 epitope.
27. The method of Claim 26 wherein said NS1 epitope stimulates a cytotoxic T cell response.
28. The method of Claim 26 wherein said NS1 epitope stimulates a helper T cell response.
- 25 29. The method of Claim 25 wherein said NS1 protein derivative consists essentially of amino acids 1-167 of NS1 protein.

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30. The method of Claim 25 wherein said NS1 protein derivative consists essentially of amino acids 1-40 of NS1 protein.
- 5 31. An essentially pure influenza A virus NS1 protein derivative in which amino acids have been deleted, inserted or substituted on the NS1 protein without essentially detracting from the immunological properties thereof wherein said NS1 protein derivative stimulates a T cell response against an NS1 epitope.
- 10 32. An essentially pure oligopeptide of Claim 31 consisting essentially of a cytotoxic T cell epitope.
33. An essentially pure oligopeptide of Claim 31 consisting essentially of a helper T cell epitope.
- 15 34. An essentially pure oligopeptide of Claim 31 consisting essentially of amino acids 1-167 of the NS1 protein.
35. An essentially pure oligopeptide of Claim 31 consisting essentially of amino acids 1-40 of the NS1 protein.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K15/02 A61K39/145

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 02250 (UNIVERSITY OF MASSACHUSETTS MEDICAL CENTER) 20 February 1992 see the whole document ---	1-35
X	EP,A,0 366 238 (SMITHKLINE BEECHAM CORPORATION) 2 May 1990 see the whole document ---	1-35
X	VIROLOGY vol. 178, no. 1, 1990, ORLANDO pages 174 - 179 KUWANO K. ET AL. 'Cross-Reactive Protection against Influenza A Virus Infections by an NS-1 Specific CTL Clone' see the whole document ----- -/--	1-35

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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Date of the actual completion of the international search

7 July 1994

Date of mailing of the international search report

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>VIROLOGY vol. 193, no. 1 , March 1993 , ORLANDO pages 289 - 295 COSSINS J. ET AL. 'Precise Prediction of a K-restricted Cytotoxic T Cell Epitope in the NS1 Protein of Influenza Virus Using an MHC Allele-Specific Motif' see the whole document ---</p>	1-35
X	<p>THE JOURNAL OF GENERAL VIROLOGY vol. 73, no. 6 , June 1992 , READING pages 1339 - 1343 HACKETT C.J. ET AL. 'Influenza virus infection elicits class II major histocompatibility complex-restricted T cells specific for an epitope identified in the NS1 non-structural protein' see the whole document ---</p>	1-35
P,X	<p>WO,A,93 15763 (SMITHKLINE BEECHAM CORPORATION) 19 August 1993 see the whole document ---</p>	1-35
A	<p>EP,A,0 176 493 (SMITHKLINE BEECHAM CORPORATION) 2 April 1986 see the whole document ---</p>	1-35
A	<p>VIROLOGY vol. 160 , 1987 , ORLANDO pages 336 - 345 SMITH G.L. ET AL. 'Synthesis and Cellular Location of the Ten Influenza Polypeptides Individually expressed by Recombinant Vaccinia Viruses' see the whole document -----</p>	1-35

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 1-12 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ At all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		CA-A-	2090005	09-02-92
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		JP-A-	3130082	03-06-91

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EP-A-0176493	02-04-86	AU-B-	592954	01-02-90
		AU-A-	4668585	06-03-86
		JP-A-	61069731	10-04-86
